Result Page

"N-DESOXYRIBOSYLTRANSFERASES OF LACTOBACILLES, SEQUENCED NUCI, EOTIDIQUES CORRESPONDING AND THEIR APPLICATIONS "

The present invention is relative with the field of the biology, and particularly of the microbiological production of analogues of bases. The present invention relates to new polypeptides and their fragments, isolated of Lactobacillus, having at least a N-désoxyribosyltransférase activity, the polynucléotides coding the aforementioned polypeptides, the vectors of cloning and/or expression including the aforementioned polynucléotides, the cells transformed by the aforementioned vectors and the specific antibodies directed against the aforementioned polypeptides. The invention also relates to an enzymatic process of synthesis of désoxyribonucléosides.

The nucleoside analogues of which the structure comprises deteriorations of sugar or heterocyclic base, train a family of active molecules in the treatment of numerous infections bacterial, viral, parasitic and fungal like in antitumor chemotherapy [Périgaud and coll, 1992]. In addition the insecticidal and herbicidal properties of certain antibiotic nucleosidic make of them potential agents in the sector of agrochemicals and the environment [Isono, 1988].

Industry employs two producing modes of these analogues, the organic synthesis and the biocatalytic conversion (enzymatic conversion and microbiological conversion), which present opposite benefits and disadvantages. The organic synthesis gives access the chemical structures most varied but requires multiple and expensive steps into reactive and solvents. On the contrary, the biocatalytic methods allow a production easy in aqueous medium but limited to a small number of possible compounds because of specificity of the enzymes, which admit a range limited of analogues to the place of their physiological substrates. The nucleosides phosphorylases and N-désoxyribosyltransférase, which come from the ways of safeguard of purins and pyrimidins in the bacteria, are the enzymes most used for these enzymatic conversions (Krenisky and coll, 1981).

There is thus an urgent need to obtain enzymes of nucleoside conversion and their derivatives, having an enzymatic activity widened in order to diversify the industrial production of these compounds.

It is the technical problem which proposes to solve the inventors of the present invention.

N-désoxyribosyltransférase de Lactobacillus leichmanii like that of L. helveticus, partially purified or not, proves to be the good donor of grouping glycosyl and tolerates a substantial number of structural variations on the base.

This enzyme was used to produce a certain number of analogues among which it is advisable to quote the 2-chloro, 2 ' - désoxadénosine (Carson and coll, 1984), 2 ', 3 ' - didésoxynucléosides of the natural bases (Carson and Wasson, 1988) or several pyrazolo (3,4-d) pyrimidin and triazolo (4,5-d) pyrimidin derived from the 2 ', 3 ' - didésoxycytidine and of corresponding base (Fischer and coll, 1990).

In the purpose having recombinant enzymes capable to treat the broadest variety of deviating substrates is by the base or by sugar, the inventors have isolated genes coding for an activity
N-désoxyribosyltransférase of different stocks of lactobacilles. This variety of Ndésoxyribosyltransférase enzymes makes it possible to increase the chances to obtain enzymes with the specificity widened by mutations in wild genes or by dreams of these wild genes.

Two classes of N-désoxyribosyltransférase were distinguished (Danzin and Cardinaud, 1976), the first (class I) indicated ptd (for purin transdésoxyribosylase) catalysing exclusively the exchange of désoxyribose between two purins: Dr.-pure + Pur' < - > Dr.-Pur'+ Pur and the second (class II) designated ntd (for nucleoside transdésoxyribosylase), the exchange of désoxyribose between a purin and a pyrimidin, two pyrimidins or two purins: Dr.-Pyr + Pure < - > Dr.-pure + Pyr Dr.-Pyr + Pyr' < - > Dr.-Pyr'+ Dr.-Pure Pyr + Pur' < - > Dr.-Pur'+ Pur
Single two genes specifying of the enzymes of class
II, indicated ntd, was to date brought back (Hück, 1997; dbjIBRA92683. 21 (AB039914)).

The present invention thus has as an object a polypeptide isolated or purified of Lactobacillus having at least a N-désoxyribosyltransférase activity of sequence of amino acids selected among sequences SEQ ID N2,
SEQ ID NR 4, SEQ ID NR 6, SEQ ID NR 8, SEQ ID NR 10, SEQ ID NR 12, SEQ ID NR 14.

According to a mode prefered of realization, polypeptide according to the invention is Ndésoxyribosyltransférase of SEQ ID N2 (or SEQ ID NR 14) coded by the gene ntd Lh de Lactobacillus helveticus.

According to a second mode prefered of realization, polypeptide according to the invention is Ndésoxyribosyltransférase of SEQ ID NR 4 coded by the gene ptd Lh de Lactobacillus helveticus.

According to a third mode prefered of realization, polypeptide according to the invention is Ndésoxyribosyltransférase of SEQ ID NR 6 coded by the gene ntd Lf de Lactobacillus fermentum.

According to a fourth mode prefered of realization, polypeptide according to the invention is Ndésoxyribosyltransférase of SEQ ID NR 8 coded by the gene ntd of Lactobacillus crispatus.

According to a fifth mode prefered of realization, polypeptide according to the invention is Ndésoxyribosyltransférase of SEQ ID NR 10 coded by the gene ntd of Lactobacillus amylovorus.

According to a sixth mode prefered of realization, polypeptide according to the invention is Ndésoxyribosyltransférase of SEQ ID? 12 coded by the gene ntd of Lactobacillus acidophilus.

Isolated polypeptide according to the invention is characterized in that it includes/understands a polypeptide chosen among (A) a polypeptide

top

of sequence SEQ ID N2, SEQ ID
NR 4, SEQ ID NR 6, SEQ ID NR 8, SEQ ID NR 10, SEQ ID NR 12,
SEQ ID NR 14; (b) a varying polypeptide polypeptide of sequences of amino acids defined in A); (c) an homologous polypeptide with defined polypeptide in (A) or (b) and comprising at least 80% of identity, preferably 85%, 87%, 90%, 95%, 97% 98%, 99% of identity with the aforementioned polypeptide of A); (D) a fragment of at least 15 consecutive amino acids preferably 17,20, 23,25, 30,40, 50,100, 250 consecutive amino acids of a defined polypeptide in A), b) or c); and (E) a biologically active fragment of a defined polypeptide in A), b) or c).

Polypeptide according to the invention is characterized in that it makes it possible to satisfy the guanine requirement of certain bacterial stocks such PAK6 which is a stock of E. coli whose two genes of the operon guaBA, which order the conversion of IMP into XMP then into GMP, as those of the operon deoCABD which order the degradation of the désoxynucléosides, were délétés. Indeed, these stocks to survive or grow require the contribution of désoxyguanosine (DRg) in the culture medium and the presence of a Ndésoxyribosyltransférase activity of a polypeptide according to the invention to carry out the exchange: DRg + A < - > Dr.-A + G.

In present description, one will use the polypeptide term to also indicate a protein or a peptide.

One will understand by polypeptide varying the whole of mutated polypeptides which can exist naturally, in particular at being human for it, and which correspond particularly to truncations, substitutions, délétions and/or additions of residues of amino-acids.

By homologous polypeptide, one will understand to indicate polypeptides presenting, compared to the natural désoxyribosyltransférases of Lactobacillus according to the invention, certain modifications like in particular a délétion, addition or substitution of at least an amino acid, a truncation, a lengthening and/or a chimerical fusion. Among homologous polypeptides, one prefers those of which the sequence of amino acids present at least 80% of identity, preferably from at least 85%, 87%, 90%, 93%, 95%, 97%, 98%, 99% of identity with the sequences of amino acids of polypeptides according to the invention. In the case of a substitution, one or more consecutive amino acids or not consecutive, can be replaced by amino acids equivalent. The expression amino acid equivalent aim here at indicating any amino acid capable to be substituted for the one of the amino acids of the basic structure without however modifying the essential characteristics or functional properties, like their biological activities (it está-statement of désoxyribosyltransférase), of corresponding polypeptides such as the induction in vivo of antibodies capable to recognize the polypeptide whose sequence of amino acids is included/understood in the sequence of amino acids SEQ ID N2, SEQ ID NR 4, SEQ ID NR 6, SEQ ID NR 8, SEQ ID NR 10, SEQ ID NR 12, SEQ ID NR 14, or one of its fragments. These equivalent amino acids can be given is while being based on their homology of structure with the amino acids for which they are substituted, that is to say on the results of the tests of crossed biological activity to which different polypeptides are capable to give place. As example, one will mention the possibilities of substitutions capable to be carried out without it resulting a thorough modification from it from the biological activities of corresponding modified polypeptides, the replacements, for example, leucine by valin or isoleucine, of the aspartic acid by the glutamic acid, the glutamine by asparagine, arginine by the lysine etc, substitutions reverse being naturally possible in the same conditions.

By polypeptide fragment, one understands to indicate a polypeptide comprising 15 consecutive amino acids at least, preferably 17,20, 23,25, 30,40, 50, 100, 250 consecutive amino-acids. The polypeptide fragments according to the invention obtained by cleavage of the aforesaid polypeptide by a proteolytic enzyme, reactive chemical, or while placing the aforementioned polypeptide in an environment very acid make part of the invention also.

By biologically active fragment, one will understand to indicate in particular a fragment of sequence of amino polypeptide acids according to the invention presenting at least one of the characteristics or functional properties of polypeptide according to the invention, particularly in what it comprises a Ndésoxyribosyltransférase activity. Variable polypeptide, homologous polypeptide or the polypeptide fragment according to the invention have at least 10%, preferably 20%, 30,40%, 50%, 60%, 70%, 80%, 90%, 95% of the N-désoxyribosyltransférase activity.

Different known protocols of the man of the art were described to introduce mutations into polypeptides. Among those it is advisable to quote as example the reaction of polymerization in chain (PCR) in the presence of manganese (Cadwell and Al, 1992). The mutations can be introduced either of manner random-in this case the step of mutagenesis is followed of a step of sifting of mutant of interest of targeted manner. Into this last case, the mutations are preferably introduced with the level of the catalytic site of N-désoxyribosyltransférases according to the invention.

Preferably a polypeptide according to the invention is a polypeptide made up of sequence SEQ ID N2, SEQ
ID NR 4, SEQ ID NR 6, SEQ ID NR 8, SEQ ID NR 10, SEQ ID NR 12, SEQ ID NR 14 or of a sequence possessing at least 80% of identity, preferably at least 85%, 90%, 95%, 98% and 99% of identity with SEQ ID N2, SEQ ID NR 4,
SEQ ID NR 6, SEQ ID NR 8, SEQ ID NR 10, SEQ ID NR 12, SEQ ID
NR 14 after optimal alignment. By polypeptide whose sequence of amino acids presenting a percentage of identity from at least 80%, preferably from at least 85%, 90%, 95%, 98% and 99% after optimal alignment with a sequence of reference, one intends to indicate polypeptides presenting certain modifications compared to the polypeptide of reference, like in particular one or more délétions, truncations, a lengthening, a chimerical fusion, and/or one or more substitutions.

Among polypeptides of which the sequence of amino acids present a percentage of identity from at least 80%, preferably from at least 85%, 90%, 95%, 98% and 99% after optimal alignment with sequences SEQ ID N2,
SEQ ID NR 4, SEQ ID NR 6, SEQ ID NR 8, SEQ ID NR 10, SEQ ID NR 12, SEQ ID NR 14 or with one their fragments according to the invention, one prefers the variable polypeptides coded by the peptidic sequences alternatives such as previously defined, in particular polypeptides of which the sequence of amino acids present at least a mutation corresponding particularly with a truncation, délétion, substitution and/or addition of at least a residue of amino acid compared to sequences SEQ ID
N2, SEQ ID NR 4, SEQ ID NR 6, SEQ ID NR 8, SEQ ID NR 10,
SEQ ID NR 12, SEQ ID NR 14 or with one their fragments; of more prefered manner, variable polypeptides presenting at least a mutation which decreases the specificity of polypeptide according to the invention for its substrate, so that variable polypeptides according to the invention are capable to catalyse a broader variety of substrate, in order to obtain a wider range of basic analogues.

The invention also relates to a purified or isolated polynucléotide characterized in that it previously codes for a polypeptide such as defined, and preferably for a polypeptide of sequence SEQ ID N2,
SEQ ID NR 4, SEQ ID NR 6, SEQ ID NR 8, SEQ ID NR 10, SEQ ID
NR 12, SEQ ID NR 14. Of prefered manner, the polynucléotide according to the invention has sequence SEQ
ID NR 1, SEQ ID NR 3, SEQ ID NR 5, SEQ ID NR 7, SEQ ID NR 9,
SEQ ID NR 11, SEQ ID NR 13.

The polynucléotide isolated or purified according to the invention characterized in that it includes/understands a selected polynucléotide among (A) SEQ ID NR 1, SEQ ID NR 3,
SEQ ID NR 5, SEQ ID NR 7, SEQ ID NR 9, SEQ ID NR 11, SEQ ID
NR 13; (b) the sequence of a fragment of at least 15 consecutive nucleotides, preferably from at least 18, 21,24, 27,30, 35,40, 50,75,

100,150, 200 consecutive nucleotides of sequence SEQ ID NR 1, SEQ
ID NR 3, SEQ ID NR 5, SEQ ID NR 7, SEQ ID NR 9, SEQ ID NR 11,
SEQ ID NR 13; (c) a nucleic sequence presenting a percentage of identity from at least 70%, preferably from at least 75%, 80%, 85%, 90%, 95%, 98% and 99% after optimal alignment with a defined sequence in A) or b); (D) the sequence complementary or the sequence of corresponding ARN to a sequence such as defined in A), b) or c).

The polynucléotide according to the invention is characterized also in what its expression in the cells hosts, particularly the bacterial stocks such PAK6, makes it possible to satisfy the guanine requirement of the aforesaid the stock. Stock PAK6 was deposited with the CNCM on May 2, 2001 under the NR I-2664. Stock PAK6 corresponds to the bacterial stock of Escherichia coli MG 1655 délétée of two genes guaA and guaB as well as genes déoC, déoA, déoB, déoD. Stock PAK6 (AguaBA: : gm Teenager-11) is auxotrophic for guanine in minimum medium glucose.

By nucleic acid, nucleic sequence or of nucleic acid, polynucléotide, oligonucleotide, sequence of polynucléotide, nucleotidic sequence, terms which will be employed indifferently in present description, one understands to indicate an accurate sequence of nucleotides, modified or not, making it possible to define a fragment or an area of a nucleic acid, comprising or not nonnatural nucleotides, and being able to correspond as well to a double DNA strand, one
Simple DNA strand that products of transcription of the aforesaid DNA, and/or a fragment of ARN.

It must be included/understood that the present invention does not relate to the nucleotidic sequences in their natural chromosomal environment, i.e. with the natural state. They are sequences which isolated and/or were purified, i.e. they were taken directly or indirectly, for example by copy, their environment having been at least partially modified. One thus also intends to indicate the nucleic acids obtained by chemical synthesis.

By polynucléotide of complementary sequence, one understands to indicate any DNA whose nucleotides are complementary to those of SEQ ID NR 1, SEQ ID NR 3,
SEQ ID NR 5, SEQ ID NR 7, SEQ ID NR 9, SEQ ID NR 11, SEQ ID
NR 13 or of a part of SEQ ID NR 1, SEQ ID NR 3, SEQ
ID NR 5, SEQ ID NR 7, SEQ ID NR 9, SEQ ID NR 11, SEQ ID
NR 13 and whose orientation is reversed.

By percentage of identity between two sequences of nucleic acids or amino acids within the meaning of the present invention, one intends to indicate a percentage of nucleotides or residues of amino acids identical between the two sequences to be compared, obtained after good alignment, this percentage being purely statistical and the differences between the two sequences being distributed randomly and over all their length. One intends to indicate by " good alignment " or " significant alignment ", the alignment for which the given percentage of identity as hereafter is highest.

The comparisons of sequences between two sequences of nucleic acids or amino acids are traditionally carried out by comparing these sequences after to have aligned them significantly, the aforementioned comparison being carried out by segment or by fenestrate comparison to identify and compare the local areas of similarity of sequence.

The significant alignment of the sequences for the comparison can be carried out, in addition to manually, by means of the algorithm of local homology of Smith and
Waterman (1981), by means of the algorithm of local homology of Neddleman and Wunsch (1970), by means of the method of search for similarity of Pearson and Lipman (1988), by means of computerised software using these algorithms (GAP, BESTFIT, BLAST P, BLAST NR, FASTA and TFASTA in Wisconsin Genetics Software Package,
Genetics Group Computer, 575 Science Dr., Madison, WI).

In order to obtain significant alignment, one uses program BLAST preferably, with matrix BLOSUM 62. One can also use matrixes WFP or PAM250.

The percentage of identity between two sequences of nucleic acids or amino acids is given by comparing these two significantly aligned sequences, the sequence of nucleic acids or amino acids to compare being able to include/understand additions or délétions compared to the sequence of reference for a significant alignment between these two sequences. The percentage of identity is calculated by determining the number of identical positions for which the nucleotide or the residue of amino acid is identical between the two sequences, by dividing this number of identical positions by the total number of compared positions and into multiplying the result obtained by 100 to obtain the percentage of identity between these two sequences.

By nucleic sequences presenting a percentage of identity from at least 70%, preferably from at least 75%, 80%, 85%, 90%, 95%, 98% and 99% after significant alignment with a sequence of reference, one intends to indicate the nucleic sequences presenting, compared to the nucleic sequence of reference, certain modifications like in particular a délétion, a truncation, a lengthening, a chimerical fusion, and/or a substitution, particularly punctual, and of which the present nucleic sequence at least 70%, preferably at least 75%, 80%, 85%, 90%, 95%, 98% and 99% of identity after significant alignment with the nucleic sequence of reference. They are preferably sequences of which the complementary sequences are capable hybrider specifically with sequences SEQ ID NR 1,
SEQ ID NR 3, SEQ ID NR 5, SEQ ID NR 7, SEQ ID NR 9, SEQ ID NR 11, SEQ ID NR 13 of the invention. Preferably, the specific conditions of hybridization or strong stringency will be such that they ensure at least 70%, preferably at least 75%, 80%, 85%, 90%, 95%, 98% and 99% of identity after significant alignment between one of the two sequences and the sequence complementary to the other one. A hybridization under conditions of strong stringency means that the conditions of temperature and ionic force are selected in such a way that they allow the maintaining of hybridization between two complementary DNA fragments. On a purely illustrative basis, conditions of strong stringency of the step of hybridization to fine of defining the fragments polynucleotidic described above, are advantageously the following ones: hybridization ADN-ADN or
ADN-ARN is carried out in two steps: (1) prehybridation at 42 C pendent 3 hours out of plug phosphates (20 mm, pH 7,5) containing 5 X SC (1 X SC corresponds to a solution 0,15 M NaCl + 0,015 M citrate of sodium), formamide 50%, sodium dodecyl 7% sulphates (SDS), 10 X Denhardt' S, 5% of dextran sulphates and DNA 1% of salmon sperm; (2) hybridization itself pendent 20 hours at a temperature dependent of the size of the probe (i.e.: 42 C, for a probe of size > 100 nucleotides) followed by 2 washings 20 minutes at 20 C in 2 X SC + 2% SDS, 1 20 minutes washing at 20 C in 0,1 X SC + 0,1% SDS. Last washing is practised in 0,1 X SC + pendent 0,1% SDS 30 minutes at 60 C for a probe of size > 100 nucleotides. The conditions of hybridization of strong stringency described above for a polynucléotide of defined size, can be adapt by the specialist of the profession for oligonucleotides of larger or smaller size, according to the teaching of Sambrook and Al, 1989.

Among the nucleic sequences presenting a percentage of identity from at least 70%, preferably from at least 75%, 80%, 85%, 90%, 95%, 98% and 99% after significant alignment with the sequence according to the invention, one prefers the nucleic sequences also alternatives of SEQ ID NR 1, SEQ ID NR 3, SEQ ID NR 5, SEQ ID NR 7, SEQ ID NR 9, SEQ ID NR 11, SEQ ID NR 13, or their fragments, i.e. the whole of the nucleic sequences corresponding with variable Al, i.e. individual variations of sequences SEQ ID NR 1, SEQ ID NR 3, SEQ ID NR 5, SEQ ID NR 7, SEQ ID NR 9, SEQ ID NR 11, SEQ ID NR 13.

Particularly, the invention relates to a nucleic acid purified or isolated according to the present invention, characterized in that it includes/understands or is consisted of the one of sequences SEQ ID NR 1, SEQ ID NR 3, SEQ ID NR 5, SEQ ID NR 7, SEQ ID NR 9, SEQ ID NR 11, SEQ ID NR 13 their sequences complementary or sequences of the corresponding ARN to SEQ ID NR 1, SEQ ID NR 3, SEQ ID NR 5, SEQ ID NR 7, SEQ ID NR 9, SEQ ID NR 11, SEQ ID NR 13.

The starters or probes, characterized in that they include/understand a sequence of a nucleic acid according to the invention, make part of the invention also.

Thus, the starters or the probes according to the invention are useful for detection, the identification, the proportioning or the amplification of sequence of nucleic acid. In particular, they can make it possible to put in evidence or to discriminate the nucleic sequences alternatives, or to identify eukaryotic or procaryotic, bacterial gene sequence the genomic new particularly, and accurately of Lactobacillus bacteria, coding for one
N-désoxyribosyltransférase, by particularly using a method of amplification such as method PCR, or a related method. According to the invention, the polynucléotides being able to be used like probe or as starts in processes of detection, identification, proportioning or nucleic amplification of sequence, have a minimum size of 10 bases, preferably from at least 15,18, 20,25, 30,40, 50 bases. According to an embodiment, the starters according to the invention are selected among sequences SEQ
ID NR 15 and SEQ ID NR 16.

The polynucléotides according to the invention can thus be used as start and/or probe in processes implementing particularly the technical one of PCR (amplification in chain by polymerase) (Rolfs and Al, 1991). This technical requires the choice of oligonucleotidic pairs of primer framing the fragment which must be amplified. One can, for example, to refer to technical described in the American patent U. S. NR 4 683.202. The amplified fragments can be identified, for example after an electrophoresis in gel of agarose or polyacrylamide, or after technical chromatographic like filtration on freezing or the exchanging chromatography of ions, then sequences. The specificity of amplification can be controlled by using like starters the nucleotidic sequences of polynucléotides of the invention and like matrixes, of the plasmides containing these sequences or the derived products of amplification. The amplified nucleotidic fragments can be used like reactive in reactions of hybridization in order to putting in evidence the presence, in a biological sample, of a target nucleic acid of sequence complementary to that of the aforesaid amplified nucleotidic fragments. The invention also aims the nucleic acids capable to be obtained by amplification using starters according to the invention.

The other technical ones of amplification of the target nucleic acid can advantageously be employed like alternative to the PCR (PCR-like) using couple of nucleotidic starters of sequences according to the invention.

By PCR-like one understands to indicate all the methods implementing direct or indirect reproductions of the sequences of nucleic acids, or else in which the systems of labelling were amplified, these technical is of course known. In general it is about the amplification of DNA by a polymerase; when the sample of origin is a ARN it is previously advisable to carry out a transcription transfers. There exists currently of very numerous processes allowing this amplification, such as for example the technical SDA (Strand Displacement Amplification) or technical of amplification to displacement of strand (Walker and Al, 1992), the technical one HEAP (Transcription-based System Amplification) described by Kwoh and Al (1989), technical the 3SR (Coil-Sustained Replication sequence) described by Guatelli and Al

(1990), the technical NASBA (Nucleic Acid Based Sequence Amplification) described by Kievitis and Al (1991), the technical TMA (Transcription Mediated Amplification), the technical LCR (Ligase Chain Reaction) described by Landegren and Al (1988), the technical one of RCR (Repair Chain Reaction) described by Segev (1992), the technical one CPR (Honest Cycling Reaction) described by Duck and Al

(1990), the technical one of amplification in Q-p-réplicase described by Miele and Al (1983). Some of these technical have had for summer sophisticated.

If the polynucléotide target to be detected is ARNm, one uses advantageously, previously with the carrying in work of a reaction of amplification using the starters according to the invention or with the carrying in work of a process of detection using the probes of the invention, an enzyme of the reverse transcriptase type in order to obtain a CDNA starting from ARNm contained in the biological sample. The CDNA obtained will be used then as target for the starters or the probes carrying works about it in the process of amplification or detection according to the invention.

The technical one of hybridization of probes can be carried out various manners (Matthews and Al, 1988).

The most general method consists in immobilizing the nucleic acid extracted the cells of different fabrics or cells in culture on a support (such as nitrocellulose, the nylon, polystyrene) to carry out for example chips with DNA, then to incubate, under else defined conditions, the target nucleic acid immobilized with the probe. After hybridization, the excess of probe is eliminated and the formed hybrid molecules are detected by the suitable method (measuring of the radioactivity, fluorescence or the bound enzymatic activity to the probe).

According to another mode of carrying in work of the nucleic probes according to the invention, these last can be used like probes of capture. In this case, a probe, said probe of capture, is immobilized on a support and is used to capture by specific hybridization the target nucleic acid obtained starting from the biological sample to test and the target nucleic acid is then detected thanks to one second probe, said probe detection, labeled by a readily detectable element.

Among the fragments of interesting nucleic acids, it is in addition advisable to quote in particular the oligonucleotides anti-direction, i.e. of which the structure ensures, by hybridization with the target sequence, an inhibition of the expression of the corresponding product. It is also necessary to quote the oligonucleotides feel which, by interaction with proteins implied in the regulation of the expression of the corresponding product, will induce either an inhibition, or an activating of this expression. Oligonucleotides according to the present invention a minimum size of 9 bases, preferably from at least 10,12, 15,17, 20,25, 30,40, 50 bases.

The probes, starters and oligonucleotides according to the invention can be labeled directly or indirectly by a or not radioactive radioactive compound, by else known methods of the specialist of the profession, in order to obtain a detectable and/or quantifiable signal. The sequences of polynucléotides according to the invention nonlabeled can be used directly like probe or starts.

The sequences are generally labeled to obtain sequences operable for numerous applications. The labelling of the starters or the probes according to the invention is carried out by radioactive elements or nonradioactive molecules. Among the radioactive isotopes used, one can quote the 32P, the 33P, the 35S, the 3H or the 125I. The nonradioactive entities are selected among the ligands the such biotin, the avidin, the streptavidine, the dioxygénine, haptens, them dyes, the luminescent agents such as the agents radioluminescent, chémoluminescents, bioluminescent, fluorescent, phosphorescent.

The invention also includes/understands a detection method and/or of proportioning of a polynucléotide according to the invention, in a biological sample, characterized in that it comprises the following steps: (I) of insulation of DNA starting from the biological sample to analyze, or obtaining of a CDNA starting from the ARN of the biological sample (II) of specific amplification of coding DNA for polypeptide according to the invention using starters; (III) of analysis of the products of amplification. It is also an object of the invention to provide a case for the detection and/or the proportioning of a nucleic acid according to the invention, in a biological sample, characterized in that it includes/understands the following elements: (I) a couple of nucleic starters according to the invention, (II) the reactive necessary ones to carry out a reaction of DNA amplification, and optionally (III) a component allowing to check the sequence of the fragment amplified, particularly a probe according to the invention.

The invention includes/understands also a detection method and/or of proportioning of nucleic acid according to the invention, in a biological sample, characterized in that it comprises the following steps: (I) of carrying in contact of a polynucléotide according to the invention with a biological sample; (II) of detection and/or proportioning of hybrid formed between the aforementioned polynucléotide and nucleic acid of the biological sample. It is also an object of the invention to provide a case for the detection and/or the proportioning of nucleic acid according to the invention, in a biological sample, characterized in that it includes/understands the following elements: (I) a probe according to the invention, (II) the reactive necessary ones with the carrying in work of a reaction of hybridization, and/or if necessary, (III) a couple of starters according to the invention, as well as the reactive necessary ones with a reaction of amplification of 1 ' DNA.

Preferably, the biological sample according to the invention in which detection and proportioning are carried out is consisted a culture medium, a cellular broyat, fluid bodily, for example an human or animal serum, blood, milk.

The present invention also relates to the recombinant vectors of cloning and/or expression including/understanding a polynucléotide according to the invention and/or expressing a polypeptide according to the invention. Such a cell host is also an object of the invention.

Preferably, the vector recombinant according to the invention are: the called vector pLH2 including/understanding the polynucléotide SEQ ID NR 1 such as present in the bacterial stock deposited with the CNCM on May 30, 2001 under the NR I-2676; the plasmide pLH2 contains a fragment Alu I of 1,4 KB containing coding gene for the NR désoxyribosyltransférase of the type II of Lactobacillus helveticus CNRZ32 clone in the SmaI site of the plasmide pBAM3; the plasmide pLH2, which expresses this enzyme, is propagated in auxotrophic stock PAK6 for guanine; the called vector pLH4 including/understanding the polynucléotide
SEQ ID NR 3 such as present in the bacterial stock deposited with the CNCM on May 30, 2001 under the NR I-2677; the plasmide pLH4 contains a AluI fragment of 1,6 KB containing coding gene for the NR désoxyribosyltransférase of the type I of Lactobacillus helveticus CNRZ32, clone in the SmaI site of the plasmide pBAM3. The plasmide pLH4, which expresses this enzyme, is propagated in auxotrophic stock PAK6 for guanine; the called vector pLF6 including/understanding the polynucléotide
SEQ ID NR 5 such as present in the bacterial stock deposited with the CNCM on May 30, 2001 under the NR I-2678; the plasmide pLF6 contains a AluI fragment of 1,36 KB containing coding gene for the NR désoxyribosyltransférase of the type II of Lactobacillus fermentum CIP102780T. The plasmide pLF6, which expresses this enzyme is propagated in auxotrophic stock PAK6 for guanine; the called vector pLA including/understanding the polynucléotide
SEQ ID NR 11 such as present in the bacterial stock deposited with the CNCM on June 21, 2001 under the NR I-2689 the plasmide pLA corresponds to the plasmide pSU19 is clone with the sites PstI and BamHI an insert containing coding gene for the NR désoxyribosyltransférase of the type II of Lactobacillus acidophilus CNRZ 1296. The plasmide is propagated in the stock of Escherichia coli TG-I.

The vectors according to the invention comprise the elements necessary with the expression, and particularly, preferably a promoter, signals of initiation and termination of the translation, as well as suitable areas of regulation of the transcription. They must be able to be maintained in a stable way in the cell and can optionally have particular signals specifying the secretion of translated protein.

The different signals of control are selected according to the cellular host used. For this purpose, the sequences of nucleic acid according to the invention can be inserted in vectors into autonomous replication within the host chosen, or of the vectors intégratifs of the selected host. Among the systems with autonomous replication, one uses preferably according to the cell host, of the systems of the type plasmide, cosmide, phagemide or minichromosome, or of the systems of the viral type, viral vectors particularly being able to be adenoviruses (Perricaudet and Al, 1992), retroviruses, lentivirus, poxvirus or viruses herpetic (Epstein and Al, 1992). The specialist of the profession knows technologies operable for each one of these systems.

When one wishes the integration of the sequence in the chromosomes of the cell host, one can use for example systems of the plasmidic type or viral; such viruses are, for example, the retroviruses (Temin, 1986), or the AAV (Housing, 1993).

Among the nonviral vectors, one prefers the naked polynucléotides such as naked DNA or the naked ARN according to the technical one developed by company VICAL, the artificial chromosomes of bacterium (TRAY; "bacterial artificial chromosome"), artificial yeast chromosomes (YAC, "yeast artificial chromosome") for the expression in yeast, artificial chromosomes of mouse (MAC, "foam artificial chromosome") for the expression in the murine cells and of prefered manner artificial chromosomes of man (HAC, "human artificial chromosome") for the expression in the human cells.

Such vectors are prepared according to methods' currently used by the specialist of the profession, and the clones into resulting can be introduced into a suitable host by methods standard, such as for example the lipofection, the electroporation, the thermal shock, the chemical transformation after permeabilisation of the membrane, cellular fusion.

The invention includes/understands moreover the cells hosts, particularly the eukaryotic and procaryotic cells, transformed by the vectors according to the invention. Among the operable cells with the directions of the present invention, one can quote the bacteria and yeasts.

According to a prefered embodiment of the invention, the bacterium is selected among the group made up of
Lactobacillus fermentum, Lactobacillus acidophilus,
Lactobacillus amylovorus, Lactobacillus crispatus,
Lactobacillus helveticum, Lactobacillus lactis,
Escherichia coli, Bacillus subtilis, Campylobacter pylori, Helicobacter pylori, Agrobacterium tumefacians,
Staphylococcus aureus, Thermophilus aquaticus,
Azorhizobium caulinodans, Rhizobium leguminosarum, Neisseria gonorrhoeae, Neisseria meningitis. According to a mode prefered of realization of the invention, the bacterium is Lactobacillus. According to a mode prefered, it is about: - the bacterium transformed by the plasmide pLH2 including/understanding polynucléotide SEQ ID NR 1 as deposited with the CNCM on May 30, 2001 under the NR I-2676; - the bacterium transformed by the plasmide pLH4 including/understanding polynucléotide SEQ ID NR 3 as deposited with the CNCM on May 30, 2001 under the NR e-2677; - the bacterium transformed by the plasmide pLF6 including/understanding polynucléotide SEQ ID NR 5 as deposited with the CNCM on May 30, 2001 under the NR e-2678; - the bacterium transformed by the plasmide pLA including/understanding polynucléotide SEQ ID NR 11 as deposited with the CNCM on June 21 under the NR I-2689.

According to another mode prefered of realization the bacterium is Escherichia coli. According to another embodiment of the invention, the cell is a yeast which is preferably Saccharomyces cerevisiae,
Saccharomyces millet beer, Candida albicans.

Among the cells host according to the invention, it is also advisable to quote the cells of insects, the animal or vegetal cells.

Preferably, the cell according to the invention is deprived of enzymatic activity capable to degrade the aforementioned désoxyribonucléoside precursory or the aforementioned désoxyribonucléoside obtained by bioenzymatic reaction catalysed by a polypeptide according to the invention.

Alternatively, the aforementioned cell host can be provided additional bio-enzymatic activities intended to transform the precursory désoxyribonucléoside, and/or the désoxyribonucléoside obtained by the bioenzymatic reaction catalysed by polypeptide according to the invention. Among these additional bio-enzymatic activities, it is advisable to quote the phosphorylation, sulphating, the acetylating, the succinylation, the methylation.

The sequence of coding nucleic acid for Ndésoxyribosyltransférases according to the invention is either naturally present in the aforementioned cell or is introduced into the aforementioned cell by the technical ones of recombinant DNA known of the specialist of the profession. According to a mode prefered of realization, the sequence of nucleic acid introduced into the aforementioned cell by the technical ones of the recombinant DNA and which codes for Ndésoxyribosyltransférase according to the invention is heterologous. One intends to indicate by sequence of heterologous nucleic acid, a sequence of nucleic acid which is not present naturally in the cell according to the invention.

The present invention also concerns the organisms métazoaires, vegetal or animal, preferably the mammals, except the Man, including/understanding of the aforesaid cells transformed according to the invention.

Among the animals according to the invention, one prefers the rodents, in particular the mice, the rats or rabbits, expressing at least a polypeptide according to the invention.

Cells preferably bacterial, or fungal particularly of yeast, as well as the organisms métazoaires according to the invention are operable in a production method of N-désoxyribosyltransférase according to the invention. The production method of a polypeptide of the invention in recombinant form, itself included/understood in the present invention, is characterized in that one cultivates the transformed cells, particularly the cells of the present invention, under conditions allowing the expression and optionally the secretion of a recombinant polypeptide coded by a sequence of nucleic acid according to the invention, and which 1 " one recovers the aforementioned recombinant polypeptide. Recombinant polypeptides capable to be obtained by this production method make part of the invention also. They can be presented in or not glycosylated glycosylated form and can have or not the tertiary structure of natural protein. The sequences of recombinant polypeptides can be also modified in order to improve their solubility, in particular in aqueous solvents. Such modifications are known of the specialist of the profession such as for example the délétion of hydrophobic fields or the substitution of hydrophobic amino acids by hydrophilic amino acids.

These polypeptides can be produced starting from the defined sequences of nucleic acid above, according to the technical ones of production of recombinant polypeptides known of the specialist of the profession. In this case, the sequence of nucleic acid used is placed under the signal proving allowing its expression in a cellular host.

An effective system of production of a recombinant polypeptide requires to have of a vector and a cell host according to the invention. These cells can be obtained by the introduction into cells hosts of a nucleotidic sequence inserted in a vector such as defined above, then the carrying in culture of the aforesaid cells under conditions allowing the replication and/or the expression of the transfectée nucleotidic sequence.

The processes used for the purification of a recombinant polypeptide are known of the specialist of the profession. Recombinant polypeptide can be purified starting from cellular lysates and extracts, of supernatant of the culture medium, by methods used individually or in combination, such as fractionation, the methods of chromatography, the technical ones of immunoaffinity using monoclonal or polyclonal antibodies specific, etc A prefered alternative consists in producing a recombinant polypeptide amalgamated with a protein carrying (protein d-eam). The benefit of this system is that it allows a stabilization and a reduction in the proteolysis of the recombinant product, an increase of solubility during in vitro renaturation and/or a simplification of the purification when the partner of fusion has an affinity for a specific ligand.

Polypeptides according to the present invention can also be obtained by chemical synthesis by using one of the numerous known peptidic syntheses, for example the technical ones implementing solid phases or the technical ones using partial solid phases, by condensation of fragments or a synthesis in conventional solution. The polypeptides obtained by synthesis chemical and being able to comprise corresponding nonnatural amino acids are also included/understood in the invention.

Polypeptides according to the invention make it possible to prepare monoclonal or polyclonal antibodies. It is thus also one of the objects of the present invention to provide a monoclonal or polyclonal antibody and its fragments, characterized in that they bind selectively and/or specifically a polypeptide according to the invention.

The chimerical antibodies, the humanized antibodies and the simple antibodies chain make part of the invention also. The fragments of antibody according to the invention are preferably fragments Fab, F (ab') 2, FC or Fv.

The polyclonal antibodies could be prepared, for example by immunization of an animal, in particular a mouse, with a polypeptide according to the invention associated with an adjuvant with the immune answer, then purification of the specific antibodies contained in the serum of the animals immunized on a column of affinity on which previously polypeptide having been used as antigen was secured. The polyclonal antibodies according to the invention can also be prepared by purification on a column of affinity, on which a polypeptide according to the invention was previously immobilized. The monoclonal antibodies could advantageously prepared starting from hybridomes according to the technical one be described by Kohler and Milstein in 1975.

According to a mode particular of realization of the invention, the antibody is capable to inhibit the interaction between N-désoxyribosyltransférase of the invention and its substrate in order to deteriorate the physiological function of the aforesaid polypeptide N-désoxyribosyl transférase.

The invention also relates to methods for the detection and/or the purification of a polypeptide according to the invention, characterized in that they implement an antibody according to the invention. The invention includes/understands moreover purified polypeptides, characterized in that they are obtained by a method according to the invention.

In addition, in addition to their use for the purification of polypeptides, the antibodies of the invention, in particular the antibodies monoclonal, can also be used for the detection of these polypeptides in a biological sample.

For these different uses, the antibodies of the invention could also be labeled same way which previously describes for the nucleic probes of the invention and prefered manner with labelling of an enzymatic type, fluorescent or radioactive.

The antibodies of the invention also constitute an analyzing mean of the polypeptide expression according to the invention, for example by immunofluorescence, labelling with gold, immunoconjugués enzymatic. Generally, the antibodies of the invention can advantageously be implemented in any situation where the expression of a polypeptide according to the invention, normal or mutated, must be observed, and particularly in immunocytochimy, immunohistochimy or in experiments of western blotting. Thus, a process of detection of a polypeptide according to the invention in a biological sample, including/understanding the steps of carrying in contact of the biological sample with an antibody according to the invention and of carrying in evidence of the complex formed antigen-antibody is also an object of the invention.

It is also one of the objects of the present invention to provide an enzymatic process of synthesis in vitro or in vivo of désoxyribonucléotides characterized in that it includes/understands at least a reactional step catalysed by at least N-désoxyribosyltransférase according to the invention. The process according to the invention is characterized in that the aforementioned N-désoxyribosyl transférase catalyzing the exchange of a first present nucleobase in a désoxyribonucléoside by one second nucleobase.

According to a mode prefered of realization of the invention, the aforementioned second nucleobase is selected in the group made up of bound purins by N9, of bound pyrimidins by N1, the bound azines by N1, of imidazols bound by N1, the aforementioned second nucleobases being able to carry substitutions of hydrogen to the nonbound positions. Preferably, the aforementioned second nucleobase is selected in the group made up of the 6-methyl purin, 2-amino-6-méthylmercaptopurine, 6diméthylaminopurine, 5-azacytidine, 2,6-dichloropurine, 6-chloroguanine, 6-chloropurine, 6-aza-thymine, 5fluoro-uracil, ethyl-4-amino-5-imidazol carboxylate, imidazol-4-carboxamide and 1, 2,4-triazole-3-carboxamide.

The aforementioned first nucleobase as for it, is selected preferably in the group made up of adenine, guanine, the thymine, uracil and 1 " hypoxanthine. These lists are not exhaustive, and it is obvious that natural analogues or nonnaturels of nucleobases can be employed in the present invention like substrate of N-désoxyribosyl transférase of the invention.

Optionally, the process in vivo according to the invention is characterized in that it includes/understands moreover the step to introduce into the cell host the first present nucleobase in a désoxyribonucléoside.

Optionally, the process in vivo according to the invention is characterized in that it includes/understands moreover the step to introduce into the cell host the second present nucleobase in a désoxyribonucléoside.

Optionally, the process in vivo according to the invention is characterized in that it includes/understands moreover the step to introduce into the cell host the first present nucleobase in a désoxyribonucléoside and the second nucleobase of manner simultaneous and/or shifted in time.

The désoxyribonucléosides capable to be produced in large quantity and of not very expensive manner by the method of biosynthesis according to the invention thus constitute compounds of interest intended for the preventive or curative treatment of pathologies human or animal, tumorous, viral such as the AIDS (syndrome of acquired human immunodeficiency), bacterial, parasitic or fungal.

Alternatively, these désoxyribonucléosides capable to be produced in large quantity and of not very expensive manner by the method of biosynthesis according to the invention constitutes the herbicidal ones and the insecticidal ones.

The present invention also provides a process of nutritional sifting intended to isolate from the désoxyribosyltransférases, preferably polypeptides according to the invention but also their homologous or their mutant. This first screen according to the invention includes/understands the steps: (I) (optionally) obtaining of a bacterial stock, such Escherichia coli, guanine auxotroph. Preferably this stock is incapable to push in the presence of désoxyguanosine like source of guanine. Of prefered manner, it is about stock PAK 6.

(II) transfer of Exogenous DNA, preferably in the shape of a vector of expression, in the bacterium, Exogenous DNA being capable to include/understand a sequence coding for a désoxyribosyltransférase.

(III) culture of the bacteria obtained with the step (II) on a medium containing of the désoxyguanosine.

(iv) insulation of Exogenous DNA transferred in the bacteria from the step (III) which developed on the medium containing of the désoxyguanosine.

The present invention provides a nutritional screen also to distinguish activities désoxyribosyltransférases I and II, preferably particularly to distinguish between polypeptides ntd and ptd according to the invention. This second screen includes/understands the steps of: (I) obtaining of a bacterial stock such for example Escherichia coli, auxtrophe for guanine and the thymidine. Preferably this stock is incapable to push in the presence of guanine and of thymidine. Of prefered manner, it is about stock PAK 26 (A guaBguaA: : Adeo-11 AthyA: : erm A (UDP-metE) zif9: : TnlO) is auxtrophe for methionine, guanine and the thymidine).

(II) transfer of Exogenous DNA, preferably in the shape of a vector of expression in the bacterium, Exogenous DNA being capable to include/understand a sequence coding for a désoxyribosyltransférase I or II.

(III) culture of the bacteria obtained with the step (II) on a medium containing of the désoxyguanosine then determination if the bacteria push or not. If the bacteria push, then the Exogenous DNA codes for an activity désoxyribosyltransférase II which is expressed in the aforementioned bacterium. If the bacteria do not push, then the Exogenous DNA is capable to code for an activity désoxyribosyltransférase I.

Other characteristics and benefits of the invention appear in the continuation of description with the examples represented hereafter.

EXAMPLES
1. HARDWARE AND METHODS
1.1. Stocks and conditions of culture:
The stocks of lactic bacteria used come from collection CNRZ (National Center from Research Zootechnic), Unit of Dairy Research and Genetic Applied, INRA, Jouy in Josas. They are cultivated in broth MRS (of Man and coll, J. Appl.

Bacteriol., 23: 130-135,1960) and incubated at 30 C, 37 C or 42 C according to species'. The stock of Escherichia coli TG1, supplied by Stratagène, is cultivated in LB (Luria broth base 10g/L, Agar-agar 16g/L) under agitation and at 37 C.

1.2. Preparation of 1 ' total cellular DNA of the lactic bacteria:
The cultures into fine of exponential phase are centrifuged pendent 5 minutes to 13000 G. The corresponding base with a culture of 2 ml is included in 200 J. L of YOUR (Sorting 50 mm, pH8, EDTA 10 mm, pH8, saccharose 250 mm) containing 20 pg/ml lysozyme and 50 U/ml of mutanolysine (Sigma). After a one hour incubation at 37 C, the clarification of the preparation is obtained by adding 60 pl de SDS

20%.

The extraction of the nucleic acids is carried out by adding to lysate 500 U. phenol L saturated with water, pH8, added with hydroxyquinoline 0. 1% and 100 pl of an isoamylic chloroform-alcohol mixing (24/1, V/V).

The solution is homogenized then centrifuged 10 minutes to 13000 G and ambient temperature. The great, clear phase and containing the nucleic acids is kept. On this last, the extraction is repeated three times in order to eliminate the undesirable cellular components. The phenol traces are eliminated by adding 500 l from isoamylic chloroform-alcohol to the aqueous phase. After homogenisation and centrifugation pendent 3 minutes to 15000 G and 4 C, the nucleic acids contained in the great aqueous phase are precipitated by addition of a cold volume of isopropanol. After a one hour incubation with-20 C, a centrifugation is carried out pendent 20 minutes to 15000 G and 4 C. The isopropanol is eliminated and replaced by 500 J, 1 d'éthanol 70%. Last a 10 minutes centrifugation to 15000 G and 4 C makes it possible to recover a base of nucleic acids. This one is put to dry in an evaporator and is suspended in 200 jj. L of water sterile containing 10 pl of ARNase with 10 pg/pl. After 15 minutes of incubation at 37 C to make act the enzyme degrading the ARN, 10 ul of the DNA solution are put to migrate by electrophoresis in a gel of agarose 0. 8% in order to evaluate the concentration and the quality of it.

1.3. Reaction of polymerization in chain of DNA (PCR):
The reactions of polymerization in chain (PCR) are carried out in a reactional volume of 100 J. L containing 20 to 100 ng DNA, 0,5 zip of starters, 200 J, M of the dNTPs (dATP, dCTP, dGTP, dTTP), in a plug Sorting
HCl pH 9 to 10 mm, KCl to 50 mm, MgCl2 to 1,5 mm, BSA with 0, 002% like 2,5 units of the Taq polymerase. Thirty cycles of amplification were applied (Gene Amp PCR systems 2400, Perkin Elmer). The inventors have defined two starters ntd 1 (SEQ ID NR 15) and ntd2 (SEQ ID NR 16) starting from the sequence ntd of Lactobacillus leichmanii described by Huck (personal communication):
EMI36.1

1.4. Hybridization of the Southern type:
Enzymatic restriction of DNA. The DNA total are digested by one or more enzymes of restriction. The enzymes used are: BamHI, BglII, ClaI, EcoRI,
HindIII, HpaI, NcoI, NotI, PstI, XbaI, XhoI (Bio-Lab). Digestion is carried out in a final volume of 40 p, L containing 70 U of enzyme, 4 p1 of plug NEB (Bio-Lab) 10X and 4 to 8; J. G of DNA. Incubation is carried out pendent lh30 at 37 C.

Transfer of 2 ' DNA on a membrane. The total DNA fragments resulting from enzymatic digestion are separate using a gel of agarose with 0, 7%. After migration, the freezing of agarose is placed under agitation in a solution of depurination (HCl 0,25N) pendent 30 minutes. This process thus allows the transfer of the great DNA fragments 10 kbs. After rinsing with water, the DNA is denatured while placing pendent freezing 40 minutes in a NaCl 5M solution, NaCOh 0,5M. Freezing is rinsed with water then again incubated 30 minutes in a solution of neutralizing, NaCl 1,5M, Tris HCl 0,5M; pH 7,5. The DNA are transferred by capillarity on a positively charged nylon membrane (Hybond N+, Amersham). They are élués by an upward flow of
SC 20X (citrate trisodium 0,3M; NaCl 3M; pH7). After the transfer, the DNA are secured of covalent manner on the membrane using an apparatus UV Stratalinker 2400 (Stratagene).

Preparation of the probe. The probe used is an internal fragment of the gene ntd of Lactobacillus helveticus amplified by PCR. The probe is purified using the kit
Wizard (Promega) in order to eliminate starters PCR. The concentration of probe necessary is 10 ng/p1. The labelling of DNA is carried out by using the kit of labelling ECL (Amersham). For this making, the DNA is denatured by heating 5 minutes at 100 C and immediately cooled in the ice. A volume of reactive of labelling (peroxidase) then a volume of solution of glutaraldhéhyde are added. This solution is incubated pendent 10 minutes at 37 C to fix of covalent manner peroxidase at DNA.

Hybridization and revelation. After a one hour prehybridation at 42 C in plug of hybridization, the membrane is hybridée pendent 16h at 42 C in the presence of the labeled probe. In order to eliminate the secured probe from nonspecific manner, the membrane is washed pendent 20 minutes at 42 C in two successive baths of plug: urea 6M-SDS 0, 4%-SSC 0,5X, then rinsed pendent 5 minutes in two successive baths of plug: citrate of sodium 0, 3M-NaCl 3M pH 7. The revelation is carried out by autoradiography according to the protocol of kit ECL. First reactive of revelation containing of hydrogen peroxide is reduced by secured peroxidase on the probe. Then the luminol contained in second reactive of revelation is then oxidized, producing light which impresses autoradiographic film.

1.5. Cloning of fragments PCR:
The homologous genes with ntd amplified by PCR are inserted in the plasmide vector pBluescript II SK+ of E. coli TG1 (Stratagene). This plasmide is before restricts in its single site by the enzyme EcoRV (Gibco
BRL) which creates blunt ends. The mixing of digestion is carried out in a volume of 30 pl, containing 4 p1 DNA. The fragments of DNA amplified with cloner must have their ends 5 ' and 3 ' blunt in order to allow the cloning. The preparation of the blunt ends of 50 SSL of products PCR purified using the kit Wizard (Promega) is carried out in a reactional volume of 100 SSL containing 3,6 units of DNA polymerase of the phage T4 (Bio-Lab) and 6 units of DNA polymerase I (or fragment of Klenow) (Bio-Lab), not having a exonucleasic activity 5 ' > 3'. Polymerization is made pendent 20 minutes at 11 C then the enzymes are inactivated after 10 minutes at 75 C. The DNA is then precipitated in the presence of two volumes of ethanol 100%, glycogen and sodium acetate 3M 10%, pH 4,8. The mixing is placed pendent one hour with-20 C then centrifuged 20 minutes to 15000 G. The base is rinsed with 250 pl ethanol with 70%, again centrifuged 10 minutes to 15000 G, dried in an evaporator and suspended in 20 sterile water pl.

The restricted DNA of the plasmide pBS-SK+ and the amplified fragment are put Co-to migrate on a gel of agarose to 0,7% in order to evaluate their respective concentrations: the number of molecules of the fragment with cloner must be three to four times great at that of the plasmide. The ligation is done in a volume of 10 p1 containing 60 ng insert, 26 ng of plasmide pBS-SK+ restricted, 2 units of ligase (T4 DNA ligase, Boehringer-Mannheim), pendent the night at 16 C. The products of ligation are pm dialysed on a Milipore filter 0,025 pm so as to remove salts and to thus avoid the electric arcs at the time of the electroporation.

1.6. Transformation:
Preparation of the electro-competent cells of stock TG1 of E. coli. From 5 ml of a culture of night at 37 C under agitation, 500 ml of medium LB are inoculated. The culture is placed under agitation at 37 C until reaching a D. O. = 1. It then is cooled pendent 2 hours in the centrifuged ice then pendent 10 cold minutes with 5000 rpm. The supernatant one is eliminated, the base is included in 400 ml of cold water. This preparation is centrifuged pendent 10 cold minutes and with 5000 trs/mn. The base obtained is again included in 250 ml of cold water. Following a 10 minutes centrifugation, the base is included in 25 ml of cold water then the cells is suspended in a final volume of glycerol 10% 1ml, and is divided into aliquot front to be frozen rapidly in liquid nitrogen.

Transformation by electroporation and selection of the clones. The preserved electro-competent cells a80 C are defrosted in the ice then carrying in contact with 5 pl of mixing of binding in a basin of electroporation. Gene-To pulsate (Bio-Rad) is controlled with 200 Volts, 25 MF, 250 Ohms. The cells are then subjected to the electroporation. One adds 1ml of a solution of PLOUGHSHARE (bactopeptone 20g/L, yeast extract 5g/L, NaCl 5M 2ml/L, KCl 1M 2.5 ml/L, MgCl2, 1M 10ml/L, MgS04 1M 10ml/L), containing 0. 4% of glucose to the cellular suspension which is carrying to be incubated at 37 C pendent one hour. The cells are then spread on a selective medium LB-Xgal (5-bromo-4-chloro-3-

indolylP-D-galatoside with lpg/mL) - IPTG. (isopropythio-S-D galactoside, lpg/ml) - Ampicilline (50 pg/ml), and incubated at 37 C pendent the night.

Plasmid DNA rapid extraction of the clones of E. coli recombinant by alkaline lysis. Cells of
E. coli transforms and cultivated in medium LB containing of the ampicilline (100 g/ml) is collected by centrifugation to 15000 G pendent 10 minutes at 4 C. They are resuspendues in 100 p1 of a solution Saccharose 50 mm, Sorting-HC1 25mM, pH 8, EDTA 10 mm, pH 8. The alkaline lysis and the denaturation of DNA are done by addition of 200 NaOh 0.2N p1, SDS 1%. The unit is let 1 minute at ambient temperature after have added 200 chloroform gl. Then, 150 p. L of a potassium acetate 5M solution, glacial acetic acid are added.

The unit is centrifuged pendent 15 min to 13000 G at 4 C. The aqueous phase containing DNA is precipitated in the presence of 2 volumes of pendent ethanol 100% then centrifuged 20 minutes to 13000 G at 4 C. The base is washed with the ethanol 70%, centrifuged 10 minutes to 13000 G then suspended in 30 L of sterile water containing of ARNase to 10 ng/ml.

1.7. Reverse PCR:
The reverse PCR makes it possible to amplify the areas flanking a DNA fragment of known sequence. This technical is held in three steps: Digestion of the DNA template. The DNA template is digested by one or two selected enzymes of restriction so that they do not cross in the known sequence of gene and that they make it possible to obtain a fragment of adapt size (1 to 3 KB). In order to choose an adapt enzyme, the Total DNA is separately digested by several enzymes. Then hybridizations of the type
Southern are carried out while using like probes the DNA fragment of known sequence. Digestions for which the hybrid probe with a fragment of size from 1 to 3 KB, are used for the reverse PCR. The DNA fragments obtained by digestion are made circular.

For that, 100 units of T4 DNA ligase and 100 I. L of plug of ligation are added to 4 to 8 DNA pg in a final volume of lml. The mixing of ligation is put to incubate at 15 C pendent the night. The bound DNA is then precipitated with 100 p1 of sodium acetate 3M, pH 4.8, 700 p1 dtisopropanol and 2 p1 of pendent glycogen then centrifuged 30 minutes to 13000 G, at 4 C. The base is rinsed in 300 lil ethanol 70% and is centrifuged 10 minutes to 13000 G, at 4 C. The base is included in 25 p. ultrapure water L.

Amplification of DNA made circular by using divergent starters. The reactions of polymerization in chain are carried out in a reactional volume of 100 L containing 20 to 100 ng DNA, 0, 5 M of starters, 200 pM of the dNTPs (dATP, dCTP, dGTP, dTTP), in a plug Sorting-HCl pH 9 to 10 mm, KCl to 50 mm, MgCl2 to 1,5 mm BSA to 0, 002% like 2, 5 units of the polymerase
Taq.

Amplification is carried out under the following conditions:
EMI42.1

94 C <SEP> 3 <SEP> minutes
<Tb> 94 C <SEP> 30 <SEP> seconds
<Tb> 50 <SEP> with <SEP> 60 C <SEP> (according to <SEP> <the SEP> Tm <SEP>
<Tb> starters <SEP> used) <SEP> 1 <SEP> minute <SEP> 25 <SEP> cycles
<Tb> 72 C <SEP> 3 <SEP> minutes
<Tb> (Gene Amp PCR systems 2400, Perkin Elmer).

Sequencing. Fragments PCR are purified by the kit Wizard (Promega) in order to eliminate nonincorporated oligonucleotides, salts and Taq polymerase. The séquençage is carried out using an automatic sequencer 373A (Applied Biosystems-Perkin
Elmer) by using a kit ABI PRISM Dye Terminator (Perkin Elmer), based on the incorporation of désoxynucléotides phosphate fluorescent at the time of the phase of elongation of the starters. The reactions of sequences are carried out in a reactional volume of 20 ju. L containing 30 ng DNA, 4 p. L of DyeT Mix (Perkin
Elmer Biosystems) and 0. ImM of starter.
EMI43.1

Cycle <SEP> :
<Tb> 96 C <SEP> 1 <SEP> minute
<Tb> 96 C <SEP> 10 <SEP> seconds
<Tb> 50 C <SEP> 5 <SEP> second <SEP> 25 <SEP> cycles
<Tb> 60 C <SEP> 4 <SEP> minutes <SEP> J
<Tb>
With each reaction of sequence are added 20 pi of sodium acetate 3M, pH 4.6, 50 pi of ethanol with 95% and 1 1 of glycogen. The solution is left 15 minutes to centrifuged ambient temperature then 20 minutes to 13000 G. The base is then rinsed with 250 pi of pendent ethanol 70% then centrifuged 10 minutes to 13000 G. The base is then included in 6 Ft1 of blue of sequence (formamide 83%, EDTA 8.3mM, blue dextran 2000000 (Sigma) 0. 5%). The samples are denatured pendent 3 minutes at 90 C and 3 pi are deposited on acrylamide 4% freezing.

2. A SINGLE NUTRITIONAL SCREEN FOR BOTH
CLASSES OF N-DESOXYRIBOSYLTRANSFERASE AT ESCHERICHIA
COLI.

A functional screen making it possible to select the production of guanine was established at E. coli, by délétant two genes of the operon guaBA which orders the conversion of IMP into XMP then into GMP like those of the operon deoCABD which orders the degradation of the désoxynucléosides, to give stock PAK 6. The genome of E. coli specifies an activity making it possible to convert the base G into GMP (guanine phosphoribosyltranférase coded by the gene gpt), as well as an activity making it possible to release the base G of désoxynucléoside DRg (purin nucleoside phosphorylase coded by the gene deoD, in the operon deo). The stock
PAK 6 present thus a guanine requirement (G) which could not be satisfied by the contribution of désoxyguanosine (DRg). The use of désoxyguanosine (DRg) could however be selected if a Ndésoxyribosyltransférase activity is expressed in stock PAK6, to carry out the exchange: DRg + A < - > Dr.-A + G
This exchange between two bases purins can be catalysed by the two classes of enzyme. In fact, the introduction of the gene ntd of L. leichmannii in stock PAK 6 makes it possible to meet the guanine requirement using désoxyguanosine (DRg) and of adenine (A).

3. FUNCTIONAL CLONING OF GENE PTD OF L.

HELVETICUS.

DNA fragments of size ranging between 1 and 2kb obtained by digestion partial (AluI) of L. helveticus CNRZ 32 was bound in a plasmide ColE1 (of pUC type digested by SmaI and déphosphorylé) and the mixing was used to transform stock PAK6.
The transformant clones were selected in inorganic medium glucose added with désoxyguanosine (DRg) and adenine (A) to the final concentration of 0,3mM.

One of the transformant clones proved to propagate a plasmide containing an insert ordering a Ndésoxyribosyltransférase activity of class I and deviating of the profile of restriction of the gene ntd of L. helveticus. The sequence of this insert revealed a gene specifying a polypeptide of 167 amino acids with a molecular weight of 18790.70 Daltons presenting a similarity of 28,6% with polypeptide NTD of L. leichmanii. The sequence of this gene indicated ptd deviates of that of the genes ntd making their hybridization impossible (7, 5% of identity).

Incidentally, the gene ntd of L. helveticus ordering a N-désoxyribosyltransférase activity of class II could be once again isolated among the selected transformant clones.

4. FUNCTIONAL CLONING OF GENE NTD OF L.

FERMENTUM.

The same operations of cloning and nutritional selection were carried out starting from Genomic DNA of the stock L. fermentum CIP 102980T. The selected transformant clones proved to propagate a plasmide whose inserts presenting of the similar profiles of restriction ordered a Ndésoxyribosyltransférase activity of class II. The sequence of one of these inserts revealed a gene specifying a polypeptide of 168 amino acids with a molecular weight of 18878.20 Daltons presenting a similarity of 32, 9% with polypeptide NTD of L. leichmanii and of 36, 7% with polypeptide PTD of L. helveticus. The sequence of this gene deviates of that of the genes ntd and ptd making their hybridization impossible. Polypeptide NTD of L. fermentum present a more labeled evolutive relationship for the enzyme of class I (PTD of L. helveticus) and a functional membership with class II, suggesting an early evolutive divergence in the evolution of these enzymes. Its enzymatic activity could be different those of the other species, and lend itself to the preparation of a larger nucleoside spectrum.

5. CLONING BY REVERSE PCR OF FOUR GENOA NTD.

By using oligonucleotides degenerated starting from areas of the sequence in amino acids of polypeptide NTD of L. leichmanii (Hück, 1997) an internal fragment with the gene ntd of L. helveticus was amplified. From this fragment, oligonucleotides were synthesized in order to obtain the totality of gene by reverse PCR.

From the two sequences ntd of L. leichmannii and of L. helveticus, we redefined starters consensus to isolate the genes ntd from other species of lactobacilles like L. acidophilus, L. crispatus, L. amylovorus with the same step as that described cidessus.

6. A nutritional screen to distinguish the two activities from désoxyribosyltransférases I and II.

To distinguish the two activities désoxyribosyl transférase, the Plasmid DNA of different selected colonies was extracted then used to transform stock PAK 26 auxotroph for guanine and of the thymidine. In stock PAK 26, the dTMP cannot be synthesized starting from the dUMP, because the thymidylate synthase coded by the gene thyA was inactivated. Moreover, the thymine cannot be a source of thymidine because the thymidine phosphorylase coded by the gene deoA and the uridine phosphorylase coded by gene UDP were délétés. Déoxyguanosine (DRg) and the thymine (T) will be the sources of guanine and thymidine, only if one activity N-désoxyribosyl transférase II is expressed in stock PAK 26 to catalyse the reaction of dG exchange + T X dT + G. Single the colonies expressing an activity N-désoxyribosyl transférase II can push on a medium inorganic glucose supplemented in déoxyguanosine and thymine like sources of guanine and thymidine. This second sifting for example made it possible to correlate the activity Ndésoxyribosyltransférase II (ntd) with the plasmide pLH2 including/understanding the polynucléotide SEQ ID NR 1 and coding for the enzyme ntd of lactobacillus helveticus and the activity N-désoxyribosyltransférase I (ptd) with the plasmide pLH4 including/understanding the polynucléotide SEQ ID NR 3 and coding for the enzymz ptd of lactobacillus helveticus.

Table 1: Growth of stock PAK 6 expressing or not a N-désoxyribosyltransférase activity on inorganic medium glucose (in vivo) and enzymatic activity of the corresponding crude extracts (in vitro)
EMI48.1

<SEP> in <SEP> in
<Tb> <SEP> vivo <SEP> vitro
<Tb> <SEP> WITH <SEP> G <SEP> dG <SEP> dG <SEP> + <SEP> WITH <SEP> cd. <SEP> + <SEP> T <SEP> dG <SEP> + <SEP>
WITH <SEP> cd. <SEP> + <SEP> With
<Tb> PAK <SEP> 6 <SEP> - <SEP> + <SEP> ~ <SEP> - <SEP> - <SEP> - <SEP>
<Tb> pSU19
<Tb> PAK <SEP> 6 <SEP> ntd-+ <SEP> + <SEP> + <SEP> + <SEP> +
<Tb> L
<Tb> PAK <SEP> 6 <SEP> ntd-+ <SEP> ~ <SEP> + <SEP> + <SEP> + <SEP> +
<Tb> Lh
<Tb> PAK <SEP> 6 <SEP> ptd-+ <SEP> +-+
<Tb> Lh
<Tb> PAK <SEP> 6 <SEP> ntd-+ <SEP> + <SEP> + <SEP> + <SEP> +
<Tb> Lf
<Tb> (+) growth (-) absence of growth
PAK 6: MG1655 AguaBA: : Apra, Adeo ntd Lf: coding gene for N-désoxyribosyltransférase of Lactobacillus leichmannii ntd Lh: coding gene for N-désoxyribosyltransférase of Lactobacillus helveticus ntd Lf: coding gene for N-désoxyribosyltransférase of Lactobacillus fermentum ptd Lh: coding gene for purin désoxyribosyltransférase of Lactobacillus helveticus
A: adenine; G: guanine; T: thymine; dG: désoxyguanosine; cd.: désoxycytidine

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